SPECIFICATION

TITLE OF THE INVENTION

Na_v2 CHANNEL GENE-DEFICIENT NON-HUMAN ANIMALS

Technical Field to Which the Invention Pertains

This invention relates to a non-human animal whose function of Na_v2 channel gene is deficient on its chromosome that shows salt intake behavior similar to that of wild-type animals under water-sufficient conditions and shows much more intakes of hypertonic saline compared with wild-type animals under water- and salt-depleted conditions, a protein acting as a sensor of extracellular sodium ion level, a gene that codes for said protein, and the like.

Prior Art

Voltage-dependent sodium channels as well as voltage-dependent potassium channels are known as the ion channels which play the main role in the generation and the propagation of action potential in excitable cells such as nerve cells, muscle cells, and the like. A sodium channel molecule comprises an ion-selective channel with voltage-sensors, and is consisted of α-subunits comprised of glycoprotein of 270kDa, and 1 or 2 smaller β-subunits. Voltage-dependent sodium channels are closed when the cell membrane is in resting potential (normally -70 to -90 mV), but they open when the cell membrane depolarizes and close approximately 1 msec later. Therefore, it is said that a sodium channel protein molecule has a voltage-sensor that senses the membrane potential, and opens the channel, a selective filter for filtering a sodium ion selectively, and inactivation gate.

Since the identification of a sodium channel protein α-subunit cDNA type I, II, and III in the brain (Nature 320, 188-192(1986), FEBS

Lett. 228, 187-194(1988)) by the inventors of the present invention, multiple structurally related isoforms of the α-subunit have been cloned from various tissues, forming a multigene family. In addition to the excitable cells, it has recently been found that glial cells also express voltage-sensitive sodium currents (Trends Neurosci. 19, 325-332(1996)). In situ hybridization, RT-PCR, Northern blot analysis and immunocytochemistry have clearly demonstrated the presence of brain-type I, II, III, H1, Na_s, NaCH6 and the like in glial cells (Glia 26, 92-96(1999)). However, the functional roles of these voltage-dependent sodium channels in so-called electrically inexcitable cells have not yet been delineated.

Several years ago, a partial cDNA homologous to the voltage-dependent sodium channel α-subunit was cloned from a cDNA library derived from rat astrocytes, and designated NaG (Proc. Natl. Acad. Sci. USA 89, 7272-7276(1992)). Subsequently, similar α-subunit isoforms were independently cloned from various animal species: $Na_v2.1$ from human heart (Proc. Natl. Acad. Sci. USA 89, 4893-4897(1992)), $Na_v2.3$ from a mouse arterial tumor cell line (J. Biol. Chem. 269, 30125-30131), and SCL11 from rat dorsal root ganglia which corresponds to splicing variant of NaG (FEBS Lett. 400, 183-187(1997)). From the sequence homology, it is possible to assume that they are species orthologues and to classify them into another subfamily of α-subunit of voltage-dependent sodium channels (NaCh), namely, subfamily 2 NaCh (Na_v2). Their overall amino acid sequences had less than 50 % identity with those of the previously cloned voltage-dependent sodium channels, and the sequences are characterized as rather unique even in the regions associated with ion selectivity and voltage-dependent activation and inactivation. Such regions are perfectly conserved in all other subfamily members, suggesting that the Na_v2 has specific channel properties. However, all the attempts to express functional Na_v2

channels in heterologous expression systems using such as Xenopus oocytes, CHO cells, HEK293 cells and the like have been unsuccessful, and the function of Na_v2 channels *in vivo* has been totally unknown.

NaG/SCL11 was originally thought to be one of the voltage-dependent sodium channels (NaChs) expressed in astrocytes because it was cloned from astrocytes, but subsequent in situ hybridization studies revealed that Na_v2 is expressed not in astrocytes but in Schwann cells and the spinal sensory neurons *in vivo* (Glia 21, 269-276(1997)). Relatively high levels of NaG mRNA are detected outside of the nervous system, particularly in lung and heart. In addition, RNase protection and Northern blot analyses demonstrated the presence of NaG mRNA in the central nervous system. However, it has been reported that NaG mRNA was not detectable by in situ hybridization using a non-isotopic probe except in the mesencephalic nucleus V. (Mol. Brain Res. 45, 71-82(1997)), suggesting that NaG mRNA is broadly expressed at a low level throughout the central nervous system or expressed restrictedly in specific regions in the central nervous system. The distribution of the NaG channel among these diverse tissues and cell-types including electrically inexcitable cells, suggests a role for this channel other than in action-potential generation and propagation.

An Object to be Attained by the Invention

So far, a model animal for excessive salt intake experiments showing salt intake behavior similar to that of wild-types under water-sufficient conditions and showing abnormal intakes of hypertonic saline compared with wild-types under water- and salt-depleted conditions has not been known. A protein acting as a sensor of extracellular sodium ion level, and a gene that codes for such protein have not been known as well. The object of the present invention is to provide a null mutant non-human animal showing salt intake behavior

similar to that of wild-type animals under water-sufficient conditions and showing much more intakes of hypertonic saline compared with wild-type animals under water- and salt-depleted conditions, for example, an Na_v2 gene deficient non-human animal, which is useful as said model animal of excessive salt intake experiments, a protein acting as a sensor of extracellular sodium ion level, and a gene that codes for said protein.

Means to Attain the Object

The inventors have been intensely studied the function and the role of Na_v2 channel *in vivo*, which was unknown, and generated Na_v2 channel knockout mice, then confirmed that Na_v2 channel plays a role to sense and control sodium ion level in body fluids. Subsequently, it has been found that these Na_v2 channel knockout mice show salt intake behavior similar to that of wild-types under water-sufficient conditions and show abnormal behavior such as much more intakes of hypertonic saline compared with wild-types under water- and salt-depleted conditions, and thus the present invention has been completed.

In other words, this invention relates to a null mutant non-human animal characterized in showing salt intake behavior similar to that of wild-type animals under water-sufficient conditions and showing much more intakes of hypertonic saline compared with wild-type animals under water- and salt-depleted conditions (Claim 1), the null mutant non-human animal according to claim 1, wherein the function of Na_v2 gene is deficient on its chromosome (Claim 2), the null mutant non-human animal according to claim 2, wherein the non-human animal is a rodent (Claim 3), and the null mutant non-human animal according to claim 3, wherein the rodent is a mouse (Claim 4).

This invention also relates to a gene that codes for a protein acting as a sensor of extracellular sodium ion level (Claim 5), the

gene according to claim 5, wherein the protein is comprised of amino acid sequence shown in Seq. ID No. 3, or is comprised of amino acid sequence where one or a few amino acids are deficient, substituted, or added, in amino acid sequence shown in Seq. ID No. 3 (Claim 6), the gene according to claim 5, which is comprised of DNA that contains a base sequence shown in Seq. ID No. 2 or its complimentary sequence, and a part or whole of those sequences (Claim 7), and the gene according to claim 5, which is comprised of DNA being hybridized under stringent conditions with DNA that contains a base sequence shown in Seq. ID No. 2 or its complimentary sequence, and a part of or whole of those sequences (Claim 8).

This invention relates to a protein acting as a sensor of extracellular sodium ion level (Claim 9), the protein according to claim 9, which is comprised of amino acid sequence shown in Seq. ID No. 3 (Claim 10), the protein according to claim 9, which is comprised of amino acid sequence where one or a few amino acids are deficient, substituted, or added, in amino acid sequence shown in Seq. ID No. 3 (Claim 11), a fusion protein created by combining a protein acting as a sensor of extracellular sodium ion level and a marker protein and/or a peptide tag (Claim 12), and the fusion protein according to claim 12, wherein the protein acting as a sensor of extracellular sodium ion level is the protein according to claims 10 or 11 (Claim 13).

This invention also relates to an antibody which specifically combines with a protein acting as a sensor of extracellular sodium ion level (Claim 14), the antibody according to claim 14, wherein the protein acting as a sensor of extracellular sodium ion level is the protein according to claims 10 or 11 (Claim 15), and the antibody according to claims 14 or 15, wherein the antibody is a monoclonal antibody (Claim 16).

This invention relates to a host cell which contains an

expression system that can express a protein acting as a sensor of extracellular sodium ion level (Claim 17), and the host cell according to claim 17, wherein the protein acting as a sensor of extracellular sodium ion level is the protein according to claims 10 or 11 (Claim 18).

This invention also relates to a transgenic non-human animal which excessively expresses a protein acting as a sensor of extracellular sodium ion level (Claim 19), the transgenic non-human animal according to claim 19, wherein the protein acting as a sensor of extracellular sodium ion level is the protein according to claims 10 or 11 (Claim 20), and the transgenic non-human animal according to claims 19 or 20, wherein the non-human animal is a mouse or a rat (Claim 21).

This invention relates to a method of screening a material that promotes or suppresses the function or the expression of a protein acting as a sensor of extracellular sodium ion level characterized in using a cell that expresses a protein acting as a sensor of extracellular sodium ion level, and a subject material (Claim 22), the method of screening a material that promotes or suppresses the function or the expression of a protein acting as a sensor of extracellular sodium ion level according to claim 22, wherein the cell that expresses a protein acting as a sensor of extracellular sodium ion level is the host cell according to claims 17 or 18 (Claim 23), and a method of screening a material that promotes or suppresses the function or the expression of a protein acting as a sensor of extracellular sodium ion level characterized in using the non-human animal according to any one of claims 1 to 4 or the non-human animal according to any one of claims 19 to 21, and a subject material (Claim 24).

This invention relates to a material that promotes or suppresses the function or the expression of a protein acting as a sensor of

extracellular sodium ion level characterized in being available through the screening method according to any one of claims 22 to 24 (Claim 25), a medical compound used for curing patients who need promotion of the function or enhancement of the expression of a protein acting as a sensor of extracellular sodium ion level, and containing the protein according to any one of claims 9 to 11 or the material that promotes the function or the expression of a protein acting as a sensor of extracellular sodium ion level according to claim 25 as its effective components (Claim 26), and a medical compound used for curing patients who need suppression of the function or the expression of a protein acting as a sensor of extracellular sodium ion level, and containing the protein according to any one of claims 9 to 11 or the material that suppresses the function or the expression of a protein acting as a sensor of extracellular sodium ion level according to claim 25 as its effective components (Claim 27).

Brief Explanation of Drawings

The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

Fig. 1 is a view showing a gene map of Na_v2 knockout mice of the present invention (a), the result of Southern blot technique (b), genomic PCR (c) and Western blot technique (d) of genomic DNA.

Fig. 2 is a view showing lacZ-expressing areas in embryos, dorsal root ganglia and the thoratic regions of Na_v2 knockout mice of the present invention.

Fig. 3 is a view showing lacZ-expressing areas in the brains of Na_v2

knockout mice of the present invention.

Fig. 4 is a view showing the result of the influence of dehydration on the expression of a nuclear protein, Fos, in Na_v2 knockout mice of the present invention.

Fig. 5 is a view showing the result of the influence of mouse Na_v2 channel deficiency of the present invention to water and salt intake of mice.

Fig. 6 is a view showing the result of responses to various tastant stimuli in the chorda tympani nerve of Na_v2 knockout mice of the present invention.

Fig. 7 is a view showing the result of the measurement of preference ratio for 0.3 M NaCl solution and total fluid intake of Na_v2 knockout mice of the present invention before and after 24-h dehydration.

Fig. 8 is a view showing the result of the sodium-depletion induced salt appetite test for Na_v2 knockout mice of the present invention.

Mode for Carrying out the Invention

The null mutant non-human animal of the present invention is not particularly limited, any non-human animal showing salt intake behavior similar to wild-types under water-sufficient conditions and showing much more intakes of hypertonic saline compared with wild-types under water- and salt-depleted conditions will suffice, however, the null mutant non-human animal whose function of Na_v2 genes is deficient on its chromosome is exemplified as a concrete example of such non-human animal. "Much more intakes of hypertonic saline compared with wild-type animals under water- and salt-depleted conditions" mentioned here means that, for example, in case of mice, the intake behavior in which the intake amount of 0.3 M saline after 24-h dehydration increases by 1.5-times or more, preferably by 2-times or more compared with that of the wild-type animals, preferably the littermate wild-type animals. Further, "the null mutant non-human

animal whose function of Na_v2 genes is deficient on its chromosome" means the non-human animal whose endogenous genes that code for Na_v2 are inactivated by its destruction, deficiency, substitution or the like, so that the animal has lost its function of expressing Na_v2, and a rodent such as a mouse or a rat and the like is exemplified as a concrete example of the non-human animal, however, the non-human animal is not limited to the exemplification. In following explanation, a mouse is cited as an example of the non-human animal.

Any constructing method of Na_v2 knockout mice will suffice as long as it can construct the knockout mice that have lost the function of expressing Na_v2. For instance, the following method is exemplified; the genomic DNA library of mice is screened by using cDNA that codes for rat NaG, which is the species counterpart of mice Na_v2, as a probe, then an Na_v2 gene of genomic DNA is isolated, subsequently a targeting vector is constructed by inserting a marker gene such as neo gene or the like into the exon of Na_v2, and thus constructed vector is induced to ES cells by electroporation method, then homologously recombined ES cells are selected, and germ line chimeric mice are constructed with this ES cells strain, and they are intercrossed with the wild-type mice, then the heterozygous mutant mice (F1: first filial generation) are obtained, and by intercrossing of those heterozygous mutant mice, wild-type mice, which are littermates of Na_v2 knockout mice generated according to Mendelian ratio, can be generated,.

The protein acting as a sensor of extracellular sodium ion level of the present invention is not particularly limited as long as it acts as a sensor of sodium ion level in nerve cells of the brain. For example, Na_v2 shown in Seq. ID No. 3 (GenBank accession number: L36179) or a protein comprised of amino acid sequence where one or a few amino acids are deficient, substituted, or added in amino acid sequence shown in Seq. ID No. 3 and acts as a sensor of extracellular sodium ion level,

or a recombined protein of said proteins are concretely exemplified. Said protein acting as a sensor of extracellular sodium ion level can be prepared based on the DNA sequence information and the like by publicly known methods.

The genes of the invention that code for a protein acting as a sensor of extracellular sodium ion level include the gene that codes for Na_v2 shown in Seq. ID No. 3 in the sequence listing, for instance, Na_v2 gene shown in Seq. ID No. 2, gene DNA that codes for a protein comprised of amino acid sequence where one or a few amino acids are deficient, substituted, or added in amino acid sequence shown in Seq. ID No. 3, and the DNA that hybridizes with these gene DNAs under the stringent conditions and codes for the protein acting as a sensor of extracellular sodium ion level. These genes can be prepared based on the DNA sequence information and the like, for example with genomic library of mice constructed from cell line R1, 129/SvJ mice gene library or the like, by publicly known methods.

It is also possible to obtain DNA of the target that codes for a protein acting as a sensor of extracellular sodium ion level, such as human $Na_v2.1$ (GenBank accession number: M91556), rat NaG/SCL11 (GenBank accession number: Y09164) and the like, which have the same effect as Na_v2 genes, by hybridizing DNA library derived from mice under the stringent conditions, with a base sequence shown in Seq. ID No. 2 or its complimentary sequence and a part or whole of those sequences as a probe, and subsequent isolation of DNA that hybridizes said probe. As conditions of the hybridization to obtain said DNA, for instance, a hybridization at 42 °C and a rinsing treatment in buffer including 1×SSC and 0.1 % SDS at 42 °C, and more preferably, a hybridization at 65 °C and a rinsing treatment in buffer including 1×SSC and 0.1 % SDS at 65 °C are exemplified. As factors influential in the stringency of a hybridization, there are various factors besides the above-

mentioned temperature condition, and it is possible for a person skilled in the art to realize a same stringency as the above-illustrated stringency of the hybridization by combining those various factors appropriately.

The fusion protein of the present invention is defined as a protein constructed by combining a protein acting as a sensor of extracellular sodium ion level, such as Na_v2 and the like, and a marker protein and/or a peptide tag. As a marker protein, any of conventionally known marker protein will suffice, for example, alkaline phosphatase, Fc region of antibodies, HRP, GFP and the like are concretely exemplified, and as a peptide tag of the present invention, conventionally known peptide tag such as Myc tag, His tag, FLAG tag, GST tag and the like are concretely exemplified. Said fusion proteins can be constructed by a usual method, and are useful as an investigational reagent in the field concerned such as the purification of a protein acting as a sensor of extracellular sodium ion level utilizing the affinity between Ni-NTA and His tag, the detection of said protein, the quantification of an antibody to said protein and the like.

As an antibody of the present invention that specifically combines with a protein acting as a sensor of extracellular sodium ion level, an immunospecific antibody such as a monoclonal antibody, a polyclonal antibody, a chimeric antibody, a single stranded antibody, a humanized antibody and the like are concretely exemplified. Though these antibodies can be constructed by a usual method with the above-mentioned protein acting as a sensor of extracellular sodium ion level as an antigen, a monoclonal antibody is more preferable among them because of its specificity. Said antibody that specifically combines with a protein acting as a sensor of extracellular sodium ion level, such as a monoclonal antibody or the like, is useful, for instance,

for the diagnosis of diseases caused by mutation or deficiency of Na_v2 such as chronic diseases of human caused by excessive intake of salt, and for elucidation of molecular mechanism of a protein such as Na_v2 or the like acting as a sensor of extracellular sodium ion level.

An antibody to a protein acting as a sensor of extracellular sodium ion level is developed by administering fragments containing a protein acting as a sensor of extracellular sodium ion level or its epitope, or cells that express said protein on the surface of the membrane to animals (preferably excluding human) with usual protocol. For instance, a polyclonal antibody can be prepared by immunizing a rabbit or the like with an antigen peptide as an immunogen, constructing antiserum by a usual method, and then purifying the constructed antiserum by a column in which the peptide used as the immunogen is fixed. Further, in preparation of a monoclonal antibody, any method that brings antibodies developed by cultured materials of continuous cell line, such as hybridoma method (Nature 256, 495-497, 1975), trioma method, human B-cell hybridoma method (Immunology Today 4, 72, 1983), and EBV-hybridoma method (MONOCLONAL ANTIBODIES AND CANCER THERAPY, pp. 77-96, Alan R. Liss, Inc., 1985) can be used. Followings are the explanation of the method of constructing a monoclonal antibody that specifically combines with Na_v2 derived from mice, that is, an anti-mNa_v2 monoclonal antibody, with an example of Na_v2 derived from mice as a protein acting as a sensor of extracellular sodium ion level.

The above-stated anti-mNa_v2 monoclonal antibody can be developed by cultivating an anti-mNa_v2 monoclonal antibody developing hybridoma *in vivo* or *in vitro* by a usual method. For instance, said anti-mNa_v2 monoclonal antibody is available through cultivation inside abdominal cavity of rodents, preferably mice or rats, *in vivo*, and through cultivation on a culture medium for animal cells *in vitro*. As examples of a medium for cultivating hybridoma *in vitro*, some cell culture

mediums, such as RPMI1640, MEM or the like, which contain antibiotics like penicillin, streptomycin or the like are exemplified.

Anti-mNa$_v$2 monoclonal antibodies developing hybridoma can be constructed, for example, by the following procedures. First, BALB/c mice are immunized by using Na$_v$2 derived from mice or the like, then spleen cells and mouse NS-1 cells (ATCC TIB-18) of the immunized mice are fused by a usual method, and then screened by immunofluorescence staining pattern. As methods of separating and purifying said monoclonal antibodies, any method generally used to purify a protein will suffice, and liquid chromatography such as affinity chromatography and the like are concretely exemplified.

In order to develop a single stranded antibody to a protein acting as a sensor of extracellular sodium ion level of the present invention, the preparation method of single stranded antibodies (US Patent No. 4,946,778) can be applied. Further, in order to express a humanized antibody, it is possible to use transgenic mice, other mammalian animals or the like, and to isolate and identify the clones that express a protein acting as a sensor of extracellular sodium ion level with the above-mentioned antibodies, and to purify the polypeptide by affinity chromatography. An antibody to a protein acting as a sensor of extracellular sodium ion level is useful for elucidating the molecular mechanism of a protein acting as a sensor of extracellular sodium ion level.

It is possible to analyze the function of said protein acting as a sensor of extracellular sodium ion level by using the above-mentioned antibodies such as anti-mNa$_v$2 monoclonal antibodies and the like labeled with fluorescent materials like FITC (fluorescein isothiocyanate), tetramethylrhodamine isothiocyanate or the like; with radioisotopes such as ^{125}I, ^{32}P, ^{14}C, ^{35}S, ^{3}H or the like; or with enzymes like alkaline phosphatase, peroxidase, β-galactosidase,

phycoerythrin or the like; or by using fusion proteins where anti-mNa_v2 monoclonal antibodies are fused with fluorescence emission proteins such as green fluorescent protein (GFP) or the like. Examples of the immunoassays include RIA method, ELISA method, fluorescent antibody technique, plaque method, spot method, hemagglutination reaction method, Ouchterony method and the like.

This invention relates to a host cell which contains an expression system that can express a protein acting as a sensor of extracellular sodium ion level. The gene that codes for a protein acting as a sensor of extracellular sodium ion level can be introduced into a host cell by a number of methods described in standard laboratory manuals by Davis et al. (BASIC METHODS in MOLECULAR BIOLOGY, 1986), and by Sambrook et al. (MOLECULAR CLONING: A LABORATORY MANUAL, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y., 1989), and the like. Examples of those methods include calcium phosphate transfection, DEAE-dextran-mediated transfection, transvection, microinjection, cationic liposome-mediated transfection, electroporation, transduction, scrape loading, ballistic introduction and infection. Examples of the host cells include bacterial procaryotic cells such as Escherichia coli, Streptomyces, Bacillus subtilis, Streptococcus, Staphylococcus and the like; fungous cells such as yeast, Aspergillus and the like; insect cells such as drosophila S2, spodptera Sf9 and the like; and animal or plant cells such as L cells, CHO cells, COS cells, HeLa cells, C127 cells, BALB/c3T3 cells (including mutant strains deficient in dihydrofolate reductase, thymidine kinase or the like), BHK21 cells, HEK293 cells, Bowes melanoma cells, oocytes and the like.

As the expression system, any expression system that can express a protein acting as a sensor of extracellular sodium ion level in a host cell will suffice. Examples of the expression system include

expression systems derived from chromosome, episome and virus, for example, vectors derived from bacterial plasmid, yeast plasmid, papovavirus like SV40, vaccinia virus, adenovirus, chicken pox virus, pseudorabies virus, or retrovirus, vectors derived from bacteriophage, transposon, and the combination of these, for instance, vectors derived from genetic factors of plasmid and of bacteriophage such as cosmid or phagemid. These expression systems may contain regulatory sequence that acts not only as a promoter but also as a controller of expressions.

A host cell that contains the above-mentioned expression system, cell membrane of said host cell, and a protein acting as a sensor of extracellular sodium ion level which is obtainable by the cultivation of said host cell can be used in the screening method of the present invention as hereinafter described. For example, the method of F. Pietri-Rouxel et al. (Eur. J. Biochem., 247, 1174-1179, 1997) or the like can be used as the method to obtain cell membranes, and publicly known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic-interaction chromatography, affinity chromatography, hydroxyapatite chromatography, and lectin chromatography, preferably high-speed liquid chromatography can be used to pick up said protein acting as a sensor of extracellular sodium ion level from cell cultured material and purify it. As columns used for affinity chromatography, in particular, there are columns to which an antibody to a protein acting as a sensor of extracellular sodium ion level such as an anti-Na_v2 monoclonal antibody and the like is bound, or in case that normal peptide tag is added to said protein such as Na_v2 or the like acting as a sensor of extracellular sodium ion level, there are columns to which materials having affinity to the peptide tag are bound. These proteins acting as sensors of extracellular sodium ion level can be obtained by using

these columns.

In the present invention, transgenic non-human animal that excessively expresses a gene that codes for a protein acting as a sensor of extracellular sodium ion level is defined as non-human animal that produces larger amount of a protein acting as a sensor of extracellular sodium ion level than wild-type non-human animal does. Though concrete examples of a non-human animal of the present invention include rodents, such as rabbits, mice, rats and the like, a non-human animal of the present invention is not limited to these animals.

Homozygous non-human animals generated according to Mendelian ratio include excessive expression type that excessively expresses a protein acting as a sensor of extracellular sodium ion level and the littermate wild-type, and it is possible to carry out precise comparative experiments in individual level by using the excessive expression types and the littermate wild-types of these homozygous non-human animals at the same time. Therefore, it is preferable to use animals of the same species, more preferably the littermates, as the wild-type non-human animals, in other words, the non-human animals that excessively express a gene that codes for a protein acting as a sensor of extracellular sodium ion level together in, for example, the screening hereinafter described in the present invention. The generating method of the non-human animals that excessively express a gene that codes for a protein acting as a sensor of extracellular sodium ion level will be explained below, with an example of transgenic mice of a protein acting as a sensor of extracellular sodium ion level.

The transgenic mice of a protein acting as a sensor of extracellular sodium ion level can be generated in following procedures. A transgene is constructed by fusing promoters such as chicken β-actin, mouse neurofilament, SV40 or the like, and poly A or introns such as rabbit β-globin, SV40 or the like with cDNA that codes for

a protein such as Na_v2 or the like acting as a sensor of extracellular sodium ion level. The transgene is microinjected in the pronucleus of a fertilized egg of a mouse, and the egg cell is cultured, then transplanted to the oviduct of a recipient mouse. After rearing up the recipient animal, baby mice that have the above-mentioned cDNA are selected from the mice born from the recipient animal. Thus transgenic mice can be generated. The baby mouse that has cDNA can be selected by extracting crude DNA from a tail or the like of a mouse, then carrying out methods like dot hybridization using a gene that codes for a protein acting as a sensor of extracellular sodium ion level as a probe, PCR method using a specific primer and the like.

By using a protein acting as a sensor of extracellular sodium ion level, a gene that codes for said protein, a fusion protein created by combining a protein acting as a sensor of extracellular sodium ion level and a marker protein and/or a peptide tag, an antibody to a protein acting as a sensor of extracellular sodium ion level, a host cell which contains an expression system that can express a protein acting as a sensor of extracellular sodium ion level, a non-human animal which excessively expresses a gene that codes for a protein acting as a sensor of extracellular sodium ion level, a null mutant non-human animal showing salt intake behavior similar to that of the wild-type animals under water-sufficient conditions and showing much more intakes of hypertonic saline compared with the wild-type animals under water- and salt-depleted conditions, a cell that expresses a protein acting as a sensor of extracellular sodium ion level, or the like, it becomes possible to screen a material that promotes or suppresses the function of a protein acting as a sensor of extracellular sodium ion level or a material that promotes or suppresses the expression of a protein acting as a sensor of extracellular sodium ion level. What obtained by this screening could be a suppressant, a preventive, or a remedy

for chronic diseases of human resulted from excessive intake of salt, or substances useful for diagnosis and cure of the diseases resulted from deficiency or abnormality of a protein acting as a sensor of extracellular sodium ion level or the like.

Examples of said screening methods include a method with cells that express a protein acting as a sensor of extracellular sodium ion level and a subject material; a method with null mutant non-human animals of the present invention showing salt intake behavior similar to that of the wild-type animals under water-sufficient conditions and showing much more intakes of hypertonic saline compared with the wild-type animals under water- and salt-depleted conditions or transgenic non-human animals which excessively express a gene that codes for a protein acting as a sensor of extracellular sodium ion level, and the subject material.

As a screening method with cells that express a protein acting as a sensor of extracellular sodium ion level and a subject material is, a method wherein cells that express a protein acting as a sensor of extracellular sodium ion level and a subject material are brought into contact, and then the change of the function or the expression of a protein acting as a sensor of extracellular sodium ion level is measured/evaluated is exemplified.

Concrete examples of a screening method with the null mutant non-human animals showing salt intake behavior similar to that of the wild-type animals under water-sufficient conditions and showing much more intakes of hypertonic saline compared with the wild-type animals under water- and salt-depleted conditions or the transgenic non-human animals which excessively express a gene that codes for a protein acting as a sensor of extracellular sodium ion level and the subject material include; a method wherein nerve cells obtained from the null mutant non-human animals showing salt intake behavior similar to that of the

wild-type animals under water-sufficient conditions and showing much more intakes of hypertonic saline compared with the wild-type animals under water- and salt-depleted conditions, or from the transgenic non-human animals which excessively express a gene that codes for a protein acting as a sensor of extracellular sodium ion level are brought into contact with a subject material *in vitro*, and then the change of the function or the expression of said protein is measured/evaluated; a method wherein saline is administrated to said null mutant non-human animals or to said transgenic non-human animals beforehand, then the nerve cells obtained from said non-human animals are cultured in the presence of the subject material, and the change of the function or the expression of said protein is measured/evaluated; a method wherein saline and a subject material are administrated to said null mutant non-human animals or to said transgenic non-human animals beforehand, then the change of the function or the expression of said protein in the nerve cells obtained from said non-human animals is measured/evaluated; a method wherein saline and a subject material are administrated to said null mutant non-human animals or to the transgenic non-human animals beforehand, then the change of the function or the expression of said protein in the non-human animals is measured/evaluated.

In measuring and evaluating the change of the function or the expression of a protein acting as a sensor of extracellular sodium ion level, it is preferable to evaluate/compare with the measured value of the wild-type non-human animals, in particular, of the littermate wild-type non-human animals, as control, because it offsets the dispersion caused by the difference between individuals. The function of a protein acting as a sensor of extracellular sodium ion level is to regulate sodium level of body fluid *in vivo*, that is, to act as a sensor of extracellular sodium ion level. Although concrete examples

of the change of said function include the change of the function in the sensory circuits for body fluid osmolarity, the change in preference-aversion response to the intake of water and salt and the like, the change of the function is not limited to these examples.

The medical compounds of the present invention are not limited particularly as long as the medical compounds contain said protein of the present invention that acts as a sensor of extracellular sodium ion level, or materials that promote or suppress the function of a protein acting as a sensor of extracellular sodium ion level, or materials that promote or suppress the expression of a protein acting as a sensor of extracellular sodium ion level as their effective components, and these medical compounds can be used for the treatment of the patients who need promotion of the function or enhancement of the expression of a protein acting as a sensor of extracellular sodium ion level, or the patients who need suppression of the function or the expression of a protein acting as a sensor of extracellular sodium ion level.

The present invention will be explained in detail with embodiments below, but the technological scope of the present invention is not limited to these embodiments.

[Construction of a targeting vector]

In order to construct a targeting vector, genomic fragments of mice containing protein-coding exons 1, 2 and 3 were cloned by NaG/SCL11 probe of rats. Nine independent genomic clones were isolated from mouse genomic libraries (donated by Dr. Mori, Osaka Univ., Japan) prepared from a cell line R1, by hybridization with a 446-bp fragment of rat NaG cDNA (nucleotide residues 11-456 encompassing the first three coding exons: GenBank accession number Y09164: Seq. ID No. 1). By Southern blot analysis using several restriction enzymes, it has been confirmed that all these overlapping clones were derived from a single

genomic locus. The hybridization-positive 3.2 and 3.7 kb HindIII fragments were subcloned into pBluescript II SK (-) (Stratagene) and subjected to DNA sequencing (the sequence is in GenBank under accession number AF190472: Seq. ID No. 4). The 3.2 kb fragment contained protein-coding exon 1 (13 bases of the 5' untranslated region and the first 238 bases in the mouse Na_v2 protein-coding sequence: nucleotide residues 238-490 of GenBank accession number L36179) and the 3.7 kb fragment contained exons 2 (nucleotide residues 491-609) and 3 (nucleotide residues 610-701). The DNA sequence encoding the three exons was identical to that of mouse $Na_v2.3$ cDNA cloned by Tamkun and coworkers (J. Biol. Chem. 269, 30125-30131 (1994)). The cloned genomic fragments showed identical restriction maps with mouse genomic DNA on Southern blot analysis with four restriction enzymes (BamH I, Bgl II, EcoR I and Hind III). This finding indicates that mouse $Na_v2.3$ is the species counterpart of rat NaG. Accordingly, $Na_v2.3$, NaG and SCL11 are designated as Na_v2.

In order to construct the targeting vector, the lacZ gene was so designed that it would be inserted into the protein-coding exon 1 of the mouse Na_v2 gene and that the N-terminal twenty amino-acid sequence of mouse Na_v2 would be fused with β-galactosidase. In other words, the 12.5 kb Sal I fragment containing the three exons was inserted into the Xho I site of pDT-A (Anal. Biochem. 214, 77-86 (1993)), then the Sal I-Xho I fragment of the lacZ-neo cassette was introduced into the endogenous Xho I site located in exon 1 (see Fig. 1a). This results in the expression of a protein that fuses the first 20 amino acids of the mouse Na_v2 protein with the N-terminus of β-galactosidase. The original genomic structure of mouse Na_v2 gene was not modified except for the insertion of the lacZ-neo cassette to make sure that the lacZ gene is expressed in place of the mouse Na_v2 gene in the targeted mice. An EcoR I linker was inserted beforehand into the 5' terminus of the

lacZ-neo cassette to utilize it as an exogenous restriction site in Southern blot screening analysis. Fig. 1a shows restriction maps of the targeting vector (top), mouse Na_v2 gene locus of wild-types (middle), and recombinant gene locus (bottom). In Fig. 1a, restriction sites B, Bg, E, H, and X represent BamH I, Bgl II, EcoR I, Hind III and Xho I respectively.

[Generation of Na_v2 knockout mice]

The above-mentioned filamentous shaped targeting vector was induced into ES cells (R1 cell line derived form 129/SV mouse) by an electroporation method. According to the method described in the paper (Neurosci. Lett. 247, 135-138 (1998)), neomycin-resistant ES clones were selected by G418, and screening of the targeted clones were performed. Homologous recombination was verified by Southern blot analysis using EcoR I digestion with probe 1 (see Fig. 1a; the 0.3 kb EcoR I-Xba I fragment located about 8 kb upstream from the Xho I site in exon 1). The selected clones were checked using probe 2 (the 0.6 kb Pst I fragment derived from the neo gene). The targeted locus was verified by genomic PCR using one sense primer (primer 1, ATGTTGACTTCCCCAGAGCC in the 5'-terminal region of exon 1, Seq. ID No. 5) and two antisense primers (primer 2, AACCAGGCAAAGCGCCATTC in the 5'-terminal region of lacZ, Seq. ID No. 6: primer 3, CATCTTCCAAGGGCTCTGACA in the 3'-terminal region of exon 1, Seq. ID No. 7). PCR amplification was carried out in two stages with EX-Taq polymerase (Takara) according to the manufacturer's protocol using a programmable thermal cycler (first stage; 95° C for 5 minutes, 60° C for 1 minute, 72° C for 1 minute: second stage; 95° C for 30 seconds, 60° C for 30 seconds, 72° C for 1 minute).

Two out of 98 ES clones, which were identified to have the correct homologous recombinant allele, were used for injection into eight-cell stage C57BL/6J mouse embryos. The injected embryos were cultured

in M16 medium overnight up to blastocysts, and seven to ten blastocysts were transplanted into the uterus of a recipient ICR mouse. The male chimeric mice thus obtained were mated with C57BL/6J females, and heterozygous mice (F1: first filial generation) were generated from littermates, and this heterozygous mutant mice were intercrossed subsequently in order to obtain homozygous mice, then Na_v2 deficient mice, which were produced according to Mendelian ratio, were generated.

The null mutant animals (mouse $Na_v2^{-/-}$) were healthy, fertile and apparently normal. The genotypic analysis of 458 four-week-old offspring obtained from breeding heterozygous animals showed an approximately Mendelian ratio between wild-type (29.5%, n=135), heterozygous mutant (48.2%, n=221) and homozygous mutant (22.3%, n=102) animals. This suggests that embryonic development and body functions were not significantly impaired in either heterozygous or homozygous mutant animals. It was reported that acute and transient expression of mouse Na_v2 was induced perinatally to the uterine smooth muscle (J. Biol. Chem. 269, 30125-30131 (1994), Am. J. Physiol. 270, C688-696 (1996)). It should be noted that pups were delivered normally in $Na_v2^{-/-}$ mice. The pattern of lacZ expression in the null mutants was identical to that in the heterozygous mutants except for the intensity of the expression, suggesting that deficiency of mouse Na_v2 does not affect the differentiation or viability of the mouse Na_v2-expressing cells.

Fig. 1b shows the result of Southern blot analysis in which samples of genomic DNA were derived from tails of wild-type (+/+), heterozygous (+/-), and homozygous (-/-) mice, and then membranes blotted with genomic DNA digested with EcoR I were hybridized with said probe 1 located on the 5' side of the targeting vector. The sizes for the wild-type (18kb) and recombinant (10kb) genotypes are shown on the right of Fig. 1b. Fig. 1c shows the result of genomic PCR analysis

of wild-type (+/+), heterozygous (+/-), and homozygous (-/-) mutant mice. The sizes for the wild-type (200bp) and recombinant (400bp) genotypes are shown on the right of Fig. 1c.

[Confirmation of non-expression of Na_v2 in Na_v2 knockout mice]

The mouse Na_v2 protein expression in the mutant mice was examined by Western blot analysis. Lung tissue samples of each wild-type (+/+), heterozygous (+/-), and homozygous (-/-) mice were prepared according to the methods of Knittle et al. (Am. J. Physiol. 270, C688-696 (1996)). SDS-polyacrylamide gel electrophoresis and immunoblotting were carried out according to the method described in the paper (Neurosci. Lett. 247, 135-138 (1998)). Anti-mouse-$Na_v2.3$ antiserum (a generous gift from Dr. M. Tumkun, Colorado State Univ., CO) was used at a 1:500 dilution in PBS. The immunoblots were incubated with several non-immune sera to verify that it was not non-specific combination. The results are shown in Fig. 1d. The position of Na_v2 protein (220kDa) is indicated on the right of Fig. 1d. The sodium channel gave a broad signal, since Na_v2 protein is highly glycosilated and readily aggregates even in the SDS-containing buffer. Compared with wild-type mice, about a half of the amount of mouse Na_v2 protein in heterozygous mutant ($Na_v2^{+/-}$) mice, and no mouse Na_v2 protein in homozygous mutant ($Na_v2^{-/-}$) mice was detected in lung membrane preparations. It is indicated that the allele was a null mutation because mouse Na_v2 protein was not expressed.

[Confirmation of the expression of lacZ in Na_v2 knockout mice by X-Gal staining]

Embryos were fixed by immersion in 3.5% formaldehyde in PBS for 1 hour at room temperature, and then cut midsagittally with a razor. Fig. 2a shows X-Gal staining of a whole-mount mouse Na_v2+/- embryo at embryonic day 15 (E15). Intensive β-galactosidase activity was observed in the trigeminal ganglia (pointed by the arrowhead in Fig. 2a) and dorsal root ganglia (pointed by the arrow in Fig. 2a)(see

reference photograph 1). In this E15 mouse, lacZ-expression was evident also in the lung (pointed by the asterisk). In these organs, the expression of lacZ persisted into adulthood. When dorsal root ganglia were cut into thin sections after X-Gal staining, β -galactosidase activity was detected in spinal sensory neurons with various cellular diameters. An X-Gal stained cryostat tissue section of dorsal root ganglion of postnatal day 2 $Na_v2^{+/-}$ mouse was shown in Fig. 2b (see reference photograph 1). The expression of lacZ was confined to the somata of neurons within dorsal root ganglion (nerve tracts are shown by asterisk in Fig. 2b), and not detected in axons. A similar pattern of lacZ expression was observed in tissue sections of the trigeminal ganglia. A cryostat section of adult sympathetic nerve trunk in the thoracic region is shown in Fig. 2c (see reference photograph 1). Based on the appearance, distribution, and size of the cell bodies, the numerous intensely stained cells are likely to be Schwann cells. The expression of lacZ was also observed in cardiac autonomic nerves and lingual nerves. These patterns of lacZ expression agreed well with the results of rat Na_v2 and mouse Na_v2 expressions (Proc. Natl. Acad. Sci. USA 89, 7272-7276 (1992), FEBS Lett. 400, 183-187 (1997), Glia, 21, 269-276 (1997)). This indicates that the lacZ gene expression is duly under the control of the regulatory regions of mouse Na_v2 gene. In Fig. 2c, the arrowheads identify the somata of Schwann cells, and scale bar = 50μm.

[Physiological roles of Na_v2]

In order to examine the physiological roles of mouse Na_v2, the lacZ-expression was surveyed throughout central nervous system (CNS) using brains of $Na_v2^{+/-}$ and $Na_v2^{-/-}$ mice. Postnatal animals were perfused under pentobarbital anesthesia first with PBS and then with the fixative. The fixed brains were cut coronally at 2mm thick or sagittally with a razor. Samples were rinsed twice with PBS and

incubated overnight in PBS containing 1mg/ml X-Gal, 5mM $K_3Fe(Cn)_6$, 5mM $k_4Fe(CN)_6$, 2mM $MgCl_2$, and 0.2% NP-40 at 37° C. For immunostaining, some X-Gal stained slices were cut further into coronal sections at 14μm thick with a cryostat microtome and mounted onto gelatin-coated slides. With rabbit polyclonal antibodies to anti-neurofilament 200 (Sigma, N-4142) or to anti-glial fibrillary acidic protein (GFAP) (Santa Cruz Biochemistry, sc-6170), immunostaining was performed (Neurosci. Lett. 247, 135-138 (1998)). Fig. 3 shows that mouse Na_v2 was expressed in specialized neurons and ependymal cells in the adult CNS (see reference photograph 2).

In Fig. 3, lacZ expression in the CNS of $Na_v2^{+/-}$ (Fig. 3a-e) and $Na_v2^{-/-}$ (Fig. 3f) mutant mice were shown. Fixed adult brains were cut coronally (Fig. 3a, b, d, e and f) or midsagittally (Fig. 3c) at 2mm and then stained with X-Gal. In (Fig. 3c), the skull under the brain was not removed. In (Fig. 3e), homozygous mutant mice were used for the analysis to detect the locus of low level expression. Explanations of the abbreviations in Fig. 3 are as follows: AH, anterior hypothalamic area; MH, medial habenular nucleus; ME, median eminence; OVLT, organum vasculosum laminae terminalis; MPO, medial preoptic area; DMH, dorsomedial hypothalamus; IPDM, interpeduncular nucleus of the dorsomedial part; MMR, medial part of the median raphe; NHP, neurohypophysis; SFO, subfornical organ; CX, cerebral cortex; BLA, basolateral amygdala. In (Fig. 3c), OVLT was removed from the central nervous system and attached to the skull. The coronal semi-whole-mount brains were cut 50 μm thick using cryostat microtome and then stained with anti-neurofilament polyclonal antibodies (Fig. 3g and h), anti-GFAP polyclonal antibodies (Fig. 3i), or cresyl violet (Fig. 3j). Brown signals are the site that reacted with the antibodies. The samples are AH (Fig. 3g), SFO (Fig. 3h and i) and ME (Fig. 3j). Arrowheads indicate double positive neurons. The asterisk in Fig. 3j

indicates the third ventricle. The dorsal side is toward the top of the panels. Scale bar: Fig. 3g to Fig. 3h = 30μm; Fig. 3j = 100μm.

As shown in Fig. 3, clusters of lacZ expression were limited to specific loci in the central nervous system (Fig. 3a-f): MPO, AH, DMH, IPDM, MMR, MeV, MH, ME, SFO, OVLT and NHP. ME, SFO, OVLT and NHP are known as the circumventricular organs (CVOs) having unusual dense and permeable capillary networks that facilitate secretion of substances into blood or penetration of substances into important tissue (FASEB J, 7, 678-686 (1993)). Relatively weak lacZ expression was detected in CX and BLA in $Na_v2^{+/-}$ mice. The intensity of the lacZ expression in these areas was more evident in $Na_v2^{-/-}$ mice (Fig. 3f). To examine the cell-types expressing lacZ, the brains were stained with X-Gal and cut into tissue sections with a cryostat microtome and subsequently immunostained with anti-neurofilament polyclonal antibodies or anti-glial fibrillary acidic protein (GFAP) polyclonal antibodies, or stained with cresyl violet. Most of the cells expressing lacZ were positive for neurofilament in the MPO, AH (Fig. 3g), IPDM, MMR, MH and MeV. GFAP-positive cells were negative for the lacZ expression, suggesting that astrocytes are negative for mouse Na_v2.

The distribution of lacZ-expressing cells in the CVOs was of particular interest. In the ME, the lacZ-expressing cells lined the floor of the third ventricle (Fig. 3j). This distribution corresponds to the location of nonciliated ependymal cells. These cells are thought to be tanycytes, which are characteristic cells providing a morphological connection between cerebrospinal fluid (CSF), nerve cells and blood vessels (Neuroscience 3, 277-283 (1978)). They are thought to be involved in exchange of substances between the CSF and pericapillary space. The lacZ-positive cells were sparsely distributed all over the SFO, and most of them were co-localized with

neurofilaments (Fig. 3h) and negative for GFAP (Fig. 3i). Intensive lacZ-positive cells also populated lining the entire third ventricle, suggesting that they are ependymal cells. In the NHP, the X-Gal signals were densely clustered. They are likely to correspond to so-called pituicytes (J. Exp. Biol. 139, 67-79 (1988)).

[Fos-immunohistochemistry]

Since analysis of the lacZ expression clearly demonstrated that mouse Na_v2 was expressed in the four CVOs and several nuclei in the central nervous system and that the mouse Na_v2-expressing cells were thus diverse not only in tissue distribution but also in cell types, it became difficult to obtain a unified view of channel function or property. However, the four CVOs are thought to be involved in body-fluid homeostasis (FASEB J, 7, 678-686 (1993), Annu. Rev. Physiol. 59, 601-619 (1997), Physiol. Rev. 78, 583-686 (1997), Physiol. Rev. 58, 582-603 (1978), Ann. NY Acad. Sci, 877, 258-280 (1999)). If the mouse Na_v2 channel functions in the sensory circuits for body-fluid osmolarity, it was expected that the activity and gene expression in these organs would be affected in the mouse Na_v2 mutant mice. Therefore, the effects of water deprivation on the central expression of Fos, a nuclear protein and a marker of changes in neural activity in response to the extracellular fluid balance in mice and rats, were examined as follows.

The time course of changes in Fos-immunopositive cell density in five regions of the brain (the medial preoptic nucleus (MnPO), organum vasculosum laminae terminalis (OVLT), subfornical organ (SFO), paraventricular nucleus (PVN), and supra optic nucleus (SON)) under water-sufficient and water-depleted conditions were examined with mice which were deprived of water for 0 h (n=4 for mouse $Na_v2^{+/+}$, n=4 for mouse $Na_v2^{-/-}$), 12 h (n=5 and 5), 24 h (n=6 and 7), or 48 h (n=6 and 5). The mice were perfused with the fixative as described above, and

their brains were immersed in the same fixative at 4° C overnight. Brains were cut coronally into sections at 50μm thick on a vibratome (Leica, VT1000S). Immunostaining was performed with a goat anti-Fos polyclonal antibody (Santa Cruz Biochemistry, sc-52-G) at a dilution of 1:1000 in PBS. Sections containing regions of interest were chosen and the Fos-immunopositive nuclei were enumerated. Each area was measured by using an image analysis system (KS400 attached to Axiophoto 2). The number of nuclei present per mm^2 was determined in the above-mentioned five regions of the brain. The results are shown in Fig.4 (see reference photograph 3).

Fig. 4a is typical examples of tissue sections containing the OVLT derived from wild-type (+/+) and null mutant (-/-) mice under water-sufficient or 24-h dehydrated condition. Scale bar = 200μm. In Fig. 4b, mean numbers of Fos-immunopositive cells per mm^2 in subfornical organ (SFO), supra optic nucleus (SON), paraventricular nucleus (PVN), organum vasculosum laminae terminalis (OVLT) and the median preoptic nucleus (MnPO) during water deprivation were plotted. The vertical bars indicate S. E., the asterisk shows that a significant difference ($P<0.05$) between $Na_v2^{-/-}$ and $Na_v2^{+/+}$ mice were revealed. In the water-sufficient condition, Fos-immunopositive cells were not detected in any region examined. At 12, 24 and 48 h after water deprivation, number of cells with Fos-immunopositive nuclei was remarkably increased in these regions in both $Na_v2^{-/-}$ and $Na_v2^{+/+}$ mice. However, in SFO and OVLT, approximately two-fold increases in Fos-immunopositive nuclei were observed in $Na_v2^{-/-}$ mice as compared with in $Na_v2^{+/+}$ mice. In the MnPO, PVN and SON, on the other hand, the rates of increase in Fos-immunopositive cells were comparable between the two groups.

[Behavioral analysis]

The effect of mouse Na_v2-channel deficiency on water and salt

intakes of mice was next examined. For the behavioral study, the mutant mice were backcrossed with C57 BL/6J males. As a result, it was verified that the behavior of F1 and F4 mice was identical. The preferance-aversion behavior of the homozygous-, heterozygous-mutant and wild-type littermates were measured by a 48-h two-bottle preference test. Mice were presented with a choice between distilled water and a tasting solution for 48 h in their home cage. For all behavioral studies, male mice at 12-24 weeks of age were used. They are individually housed under constant room temperature, humidity and 12/12 h light-dark cycle. The positions of the two bottles were switched every 24 h to avoid side preference. The total intake for each animal was measured and used to calculate a preference ratio according to the following formula: preference ratio = volume of tasting solution (ml) / total intake volume of tasting solution and water (ml). The results are shown in Fig. 5. Preference ratios for NaCl solutions with a series of concentrations are shown in Fig. 5a, and that for three fundamental tastants with fixed concentrations are shown in Fig. 5b. Homozygous (-/-), heterozygous (+/-) and wild-type (+/+) mice, five mice each, were used here. Fig. 5a shows that the concentration sensitivity to a series of NaCl solutions was comparable among the three groups of mice under the condition satiated with water and salt, and all the groups showed maximum preference to 0.1 M NaCl and evasiveness to 0.3 M or higher concentration of NaCl. The null mutants showed normal preferences to various tastants under the condition satiated with water and salt. Fig. 5b shows that preference ratios to sweet (0.5 M sucrose), sour (0.01 M HCl) and bitter (0.02 M quinine hydrochloride) tasters were not different among the groups.

[Electrophysiology]

To verify the normality in taste responses in the null mutants, electrophysiological analysis was performed on the chorda tympani

nerve, which is known to be the nerve fiber responsible for tasting NaCl. Male mice at 12-24 weeks of age were used (4 wild-type and 5 homozygous mice for the normal condition; 3 wild-type and 5 homozygous mice for the acute salt-appetite condition). Each mouse was deeply anesthetized by an intraperitoneal injection of sodium pentobarbital (60 mg/kg), then tracheotomized and secured with a head holder. The chorda tympani nerve was exposed, freed from surrounding tissues, and cut at the point of its entry to the bulla. The whole bundle of the nerve was dissected and lifted on a platinum wire recording electrode (0.1 mm in diameter). An indifferent electrode was attached to nearby tissue. The nerve activities were amplified, displayed on an oscilloscope, and monitored with an audioamplifier. The amplified signal was passed through an integrator with a time constant of 0.3 sec and was displayed on a slipchart recorder.

The taste solutions were 0.1 M NH_4Cl, 0.1 M NaCl, 0.5 M sucrose, 0.01 M HCl, 0.02 M Q-HCl, 0.1 M KCl and 0.1 M CH_3COONa (AcNa). These solutions were made up with distilled water and 0.1 mM amiloride solution. Each solution and rinsing water were applied to the anterior part of the tongue at room temperature ($25 \pm 2°$ C). The tongue was rinsed for at least 45 sec between successive stimuli. The magnitude of the whole nerve response was measured as the height of the integrated response from the baseline at 10 sec after onset of stimulation. Recorded results of the chorda tympani response to various taste stimuli are shown in Fig. 6a. The magnitudes of the responses to various taste stimuli expressed as relative values with the magnitude of the response of 0.1 M NH_4Cl taken as the standard are shown in Fig. 6b.

The neurophysiological responses to 0.02 M Q-HCl, and 0.1 M CH_3COONa were of similar intensity between the null-mutant and wild-type mice. Responses to 0.1 M NaCl and 0.1 M CH_3COONa were

decreased to the same degree by amiloride application in both groups of mice, indicating that amiloride-sensitive channels in taste buds in the mutant mice function normally. Similar results were observed in the null-mutant and wild-type mice under the acute salt appetite condition. This finding together with the normal behavioral response to various tastants under water- and salt-sufficient conditions (Fig. 5) shows that the taste reception of the null mutants is not impaired.

Under the water-depleted condition, animals take in a large quantity of water and avoid hypertonic saline to recover from the hypertonic state. The preference to hypertonic saline (0.3 m NaCl) before and after 24-h dehydration was examined. Before testing, mice were trained to drink water from two bottles for one week. On the day before dehydration, mice were presented with a choice between water and 0.3 M NaCl at 10.00 h, and then measured for fluid intake at 16.00 h. At 10.00 h on the next day, the bottles were removed. Dry food was placed throughout the period of water deprivation. After 24-h dehydration, the two bottles were returned and fluid intakes were measured at 16.00 h. The results are shown in Fig. 7. The null mutants showed an abnormal ingestion of hypertonic saline under the water-depleted condition. Preference ratio for 0.3 M NaCl solution (Fig. 7a) and total fluid intake (Fig. 7b) were measured before and after 24-h dehydration. In this experiment, n=6(+/+), 6(+/-) and 6(-/-). Vertical bars in the Fig. 7 indicate S.E., and the asterisk shows that a significant difference ($P<0.05$) between $Na_v2^{-/-}$ and $Na_v2^{+/+}$ mice were observed. In contrast to the wild-type and heterozygous mutant mice, which showed markedly decreased preference ratios to hypertonic saline after dehydration, the null mutants showed no change in the preference ratio (Fig. 7a). Total water intake (water plus 0.3 M NaCl) did not differ among the groups both before and after 24-h dehydration. The total water intake of all the groups showed more than a four-fold

increase after dehydration (Fig. 7b).

Blood was recovered from animals before or after dehydration by decapitation, and then the concentrations of plasma electrolytes were measured by using an electrolyte analyzer (9180, AVL Scientific, GA). The electrolyte concentrations in the serum before and after dehydration were normal in both wild-type and homozygous mutant mice (n=6 each). The electrolyte concentrations in wild-type and homozygous mutant mice before dehydration are as follows respectively: 153.6 $\pm$ 0.6 and 153.0 $\pm$ 1.2 mM for Na^+; 4.6 $\pm$ 0.1 and 4.7 $\pm$ 0.1 mM for K^+; 118.5 $\pm$ 0.6 and 118.3 $\pm$ 0.9 mM for Cl^-. The electrolyte concentrations in wild-type and homozygous mutant mice after dehydration are as follows respectively: 151.6 $\pm$ 0.8 and 150 $\pm$ 0.3 mM for Na^+; 6.5 $\pm$ 0.2 and 6.7 $\pm$ 0.2 mM for K^+; 116.0 $\pm$ 1.0 and 116.4 $\pm$ 0.8 mM for Cl^-. This suggests that the null mutants immediately excreted excessive amounts of sodium into urine, and thus the renal function of null mutants works normally.

Furthermore, motivated salt appetite was induced by intraperitoneal injection of a diuretic drug, furosemide, and sodium-depleted food, then the sodium-depletion induced salt appetite test was carried out in the following way. Before testing, control measurements of water and 0.3 M NaCl intake were performed for several days. At 10.00 h, mice were injected intraperitoneally with 0.12 ml of normal saline (0.9 % NaCl). The bottle of 0.3 M NaCl was withdrawn and sodium-depleted food was supplied in place of a normal diet. The second injection of normal saline was given at 16.00 h. On the following day, water and 0.3 M NaCl were presented at 10.00 h and intakes of 0.3 M NaCl and water were measured at 12.00, 14.00 and 16.00 h. After that, a similar protocol with furosemide injection (0.6 mg in 0.12 ml of normal saline) was performed with sodium-depleted food in the same mice (acute salt-appetite condition). Finally, the same protocol,

except that normal sodium-containing food was supplied, was performed to evaluate the effect of sodium-depleted food. The results are shown in Fig. 8.

The ingested volumes of water and 0.3 M NaCl are shown in Fig. 8 in cumulative values for every 2 h. The results of the behavioral study are shown in Fig. 8 as plotted mean cumulative intakes of 0.3 M NaCl (right) and water (left) per 2 h starting just after each experimental procedure as follows: a sodium-depleted diet combined with normal saline injection (top), a sodium-depleted diet combined with furosemide injection (middle), and a sodium-contained diet combined with furosemide injection (bottom). In this experiment, n = 10 (+/+), 10 (+/-) and 10 (-/-). Vertical bars in Fig. 8 indicate S. E., and the asterisk shows that a significant difference ($P<0.05$) between $Na_v2^{-/-}$ and $Na_v2^{+/+}$ mice were observed. As shown in Fig. 8, under the control condition in which isotonic saline was injected in place of a furosemide solution, the ingested volumes of water and 0.3 M NaCl were comparable among the three groups (graphs at the top). Under the acute salt appetite condition induced by furosemide injection with a sodium-depleted diet, however, the null-mutants showed an approximately two-fold increase in the ingestion of 0.3 M NaCl compared with the wild-type and heterozygous mutant mice (right graph at the middle). This abnormal ingestion of hypertonic saline stopped when sodium-containing food was provided (graph at the bottom).

[Na_v2 channel is a sodium concentration-dependent sodium channel]

It has been verified that Na_v2 channel is a sodium concentration-dependent sodium channel by following experiments.

First, an anti-Na_v2 antibody was constructed as follows: a peptide comprised of an amino acid sequence (SVSETVPIASGESDIK; Seq. ID No. 8), which exists in inter domain 2-3 of rat Na_v2 channel, was combined to hemocyanin, then a white rabbit was immunized with the

hemocyanin-combined peptide as an immunogen, and anti-rat Na_v2 rabbit antiserum was constructed by a usual method. The constructed antiserum was purified by a column in which the peptide used as the immunogen was fixed, and an anti-Na_v2 antibody was obtained. The specificity of the anti-Na_v2 antibody was confirmed by Western blot and immunohistochemical analysis. When the purified anti-Na_v2 antibody was used, no non-specific positive signal was observed in sections of brain, lung, dorsal root ganglia and tongue prepared from gene-deficient mice.

Next, nerve cells in dorsal root ganglia were isolated. The dorsal root ganglia were prepared from wild-type and Na_v2 gene-deficient mice of 8-16 weeks of age. Nerve cells were dispersedly isolated from the dorsal root ganglia according to the method of Renganathan et al. (J Newrophysiol 84, 710-718, 2000). Before used for an ion imaging experiment, the dispersedly isolated nerve cells were cultured under the condition of the humidity of 100% and the temperature of 37° C, and with 5% of carbon dioxide, then adhered to the glass of culture plates. All nerve cells were confirmed to be Na_v2-positive by staining nerve cells of dorsal root ganglia derived from wild-type mice with the above-mentioned anti-Na_v2 antibody. The size of the dispersedly isolated nerve cells were comprised of 3 groups of small (25 micron or smaller in diameter: about 50%), medium (25 to 40 micron in diameter: about 40%), and large (40 micron or larger in diameter: about 10%). However, there was no difference between the materials isolated from wild-type and gene-deficient mice in the size, shape and survival rate of these 3 types of cell. The survival rate was verified by Tripan blue staining.

In addition, nerve cells of subfornical organs were also isolated. The subfornical organs were prepared from wild-type and Na_v2 gene-deficient mice of 8-16 weeks of age. In order to visualize the

subfornical organs, Evans blue was intraperitoneally injected in advance. The subfornical organs were dispersed according to the method of Jurzak et al. (Brain Res 662, 198-208, 1994). As in the case of nerve cells of dorsal root ganglia, the dispersedly isolated nerve cells were cultured under the condition of the humidity of 100% and the temperature of 37° C, and with 5% of carbon dioxide, then adhered to the glass of culture plates, and then used for the experiment. Some nerve cells of subfornical organs derived from wild-type mice were stained with the above-mentioned anti-Na_v2 antibody, and a ratio of Na_v2-positive nerve cells was about 20 to 30%. There was no difference between the nerve cells isolated from wild-type and gene-deficient mice in the size and survival rate.

With regard to the nerve cells of dorsal root ganglia or subfornical organs prepared from the above-mentioned wild-type and Na_v2 gene-deficient mice, intracellular sodium ion and intracellular calcium ion were measured. SBFI/AM (sodium-binding benzofuran isophthalate acetoxymethyl ester) was used for measuring intracellular sodium ion, and Fura-2/AM was used for measuring intracellular calcium ion. Cultured cells loaded with these indicators were adhered to culture plates, and the culture plates were fixed to the stage of a microscope. Fluorescence ratio (F340/F380) was monitored by the fluorescence imaging system. In order to measure data, samplings were conducted every 10 seconds for the nerve cells of dorsal root ganglia, and every 20 seconds for those of subfornical organs respectively. Before the measurement, the nerve cells were incubated with physiological isotonic liquid (145 mM of extracellular sodium concentration) for 30 to 60 minutes, and while measuring, the nerve cells were exposed to a certain perfusate (5 mM of KCl, 2.5 mM of $CaCl_2$, 1 mM of $MgCl_2$, 10 mM of HEPES, 10 mM of NaOH, NaCl at the prescribed concentration, pH 7.4, (neutralization by HCl), and the perfusate

(extracellular liquid) was perfused at a certain speed (1 ml/min) at room temperature.

The results of the fluorescence imaging of fluorescence ratio (F340/F380) in nerve cells of dorsal root ganglia are shown in Fig. 9 and Fig. 10. As shown in Fig. 9, when the concentration of the extracellular NaCl was increased to 145 to 170 mM, the concentration of intracellular sodium ion in nerve cells of dorsal root ganglia derived from wild-type mice showed a rapid increase, and reached to steady state straightly. On the other hand, in the nerve cells of dorsal root ganglia derived from gene-deficient mice, an increase of the intracellular sodium ion concentration, such as observed in wild-type mice, was not observed at all (Fig. 9a and 9b). Further, the increase of sodium in the nerve cells of dorsal root ganglia derived from wild-type mice was observed in nerve cells of every size (Fig. 9c). This is consistent with the fact that Na_v2 expresses in every nerve cells of dorsal root ganglia of wild-type mice.

In addition, as shown in Fig. 10, the increase of the intracellular sodium ion concentration was caused not by osmotic pressure stimulus using mannitol or by single stimulus of chlorine ions using choline chloride, but by single stimulus of sodium ions using sodium methanesulfonate (Fig. 10a). Accordingly, it has been found that this phenomenon is caused only by the increase of sodium ion concentration. When the extracellular sodium concentrations were arranged to be 120, 130, 140, 150, 160, 165, 170, 180, 190 and 200 mM, 10 concentrations in all, with sodium chloride and the changes in intracellular sodium ion concentration were analyzed, there was no response in the range of 120 to 150 mM, the increase was observed in the extracellular sodium ion concentration of 160 and 165 mM, and extremely significant increase was observed in the extracellular sodium ion concentration of 170 mM and higher (Fig. 10b). In case the

extracellular sodium was rearranged to the original concentration of 145mM at that time, it was observed that the intracellular sodium concentration gradually changed to the original concentration. As there was no intracellular sodium store, it was concluded that this increase of the intracellular sodium concentration was attributed to an influx from extracellular regions via Na_v2 channel. The threshold value of the channel opening is presumed to be in the range of 160 to 170 mM.

In order to investigate the possibility that molecules other than Na_v2 channel are involved in the influx of sodium into the cells accompanied with this increase of the extracellular sodium ion concentration, the effects of each blocker of various sodium ion pumps, sodium ion transporters, sodium ion channels were examined. As a result, it was found that there was no influence of TTX-sensitive voltage-dependent sodium channels (Fig. 10c), amiloride-sensitive sodium channels (Fig. 10c), sodium glucose co-transporters, sodium calcium antiporters, sodium potassium chloride transporters and sodium potassium pumps. Further, it was concluded that TTX-nonsensitive voltage-dependent sodium channels were not involved in this phenomenon observed in cell types of every size because they expressed specifically in small nerve cells of dorsal root ganglia. The fact that sodium calcium antiporters were not involved in this phenomenon was also confirmed by calcium imaging (Fig. 10d).

The results of the fluorescence imaging of fluorescence ratio (F340/F380) in nerve cells of subfornical organs are shown in Fig. 11. As shown in Fig. 11, basically same results as in the case of the nerve cells of dorsal root ganglia are obtained also in the nerve cells of subfornical organs, which are organs for detecting sodium ion concentration in the central nervous system. About 20 to 30% of the nerve cells of subfornical organs were Na_v2 immuno-positive cells, and

an increase of the intracellular sodium ion concentration was observed in these Na_v2 immuno-positive cells, however, no increase of the intracellular sodium ion concentration was observed in Na_v2 immuno-negative cells, as in the case of Na_v2 gene-deficient mice. These results verify that Na_v2 is a sodium channel which makes sodium ion flow into cells in an extracellular sodium ion concentration-dependent manner in subfornical organs as well. In other words, it has been found that Na_v2 is a new sodium channel of a sodium concentration-dependent sodium channel.

Industrial Applicability

A null mutant non-human animal characterized in showing salt intake behavior similar to that of wild-type animals under water-sufficient conditions and showing much more intakes of hypertonic saline compared with wild-type animals under water- and salt-depleted conditions, for example, an Na_v2 channel gene-deficient mouse, is useful as a model animal of excessive salt intake experiments. By using this Na_v2 channel gene-deficient mouse, it has been revealed that Na_v2 channel acts a role to sense and control sodium ion level in cerebrospinal fluid, and that Na_v2 channel expresses in neurons and ependymal cells in restricted areas of the central nervous system, particularly in the circumventricular organs which are involved in body-fluid homeostasis, and that Na_v2 channel plays an important role in the central sensing of body-fluid sodium level and regulation of salt intake behavior.

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